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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

MAR 21 1995

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Pine Oil: Toxicity Studies (Inhalation, 81-3; Primary Eye Irritation, 81-4; Primary Dermal Irritation, 81-5; Dermal Sensitization, 81-6; and Salmonella typhimurium, 84-2(a)).

TO: Wooge/Davis, PM 52
SRRD (7508W)

FROM: Byron T. Backus, Ph.D., Toxicologist Byron T. Backus 3/15/95
Toxicology Branch 2
HED (7509C)

THROUGH: K. Clark Swentzel K. Clark Swentzel 3/17/95
Section Head, Review Section II
Toxicology Branch 2
HED (7509C)

and

Marcia van Gemert, Ph.D., Branch Chief mkvg'mcd 3/20/95
Toxicology Branch 2
HED (7509C)

DP Barcode: D210191 Submission: S478447

Chemical: 067002 Pine Oil

Action Requested:

Review of 4 acute studies: MRID 433752-08 (Inhalation LC50 in rats, 81-3); MRID 433752-09 (Primary eye irritation in rabbits, 81-4); MRID 433752-10 (Primary dermal irritation in rabbits, 81-5); MRID 433752-11 (Buehler dermal sensitization in guinea pigs, 81-6); and an Ames mutagenicity study in MRID 433752-12 (84-2(a)).

Executive Summaries:

1. In the inhalation LC50 study there were no mortalities among 10 rats (5 males, 5 females) which received a 4-hour full-body exposure to a measured concentration of 3.79 mg/liter (nominal concentration: 9.2 mg/liter) of Pine Oil. A mean of 40% (by weight) of the particles had an aerodynamic diameter <1 micron.

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Symptoms (depressed activity, yellow perineal and abdominal staining, chromorhinorrhea, ataxia, nasal discharge) were gone by two days after exposure, and respiratory tracts appeared normal at necropsy on day 14. The inhalation LC50 is greater than 3.79 mg/liter.

Pine oil is in **toxicity category IV (LC50 ≥ 2 mg/liter for 4-hr exposure)** in terms of its inhalation hazard potential, using the classification criteria in the Interim EPA Policy for Particle Size and Limit Concentration Issues in Inhalation Toxicity Studies.

The study is classified as acceptable. This study is acceptable as supporting data for purposes of registration and/or reregistration of products consisting of or containing pine oil.

2. In the primary eye irritation study, 6/6 rabbit eyes showed corneal opacity in the period between 24 hrs and 4 days after exposure to the test material, with corneal opacity still present in 2/6 eyes at 7 days, and all eyes normal by day 17.

   Pine oil is in **toxicity category II** in terms of its eye irritation potential.

   The study is classified as acceptable. This study can be used as supporting data (Guideline 81-4) for the registration and/or reregistration of products consisting of or containing pine oil as an active ingredient.

3. In the primary dermal irritation study, 0.5 ml of the test material was applied to single sites on each of 6 rabbits, with 4 hour occluded exposure. At 72 hours the mean combined score for erythema and edema was 3.33; the PDIS calculated by combining 24 and 72 hour scores was 3.67. All dermal irritation was gone by day 8; indicating no permanent dermal damage.

   Pine oil is in **toxicity category III** in terms of its dermal irritation potential.

   The study is classified as acceptable. This study can be used as supporting data (Guideline 81-5) for the registration and/or reregistration of products consisting of or containing pine oil as an active ingredient.

4. In the dermal sensitization study, 10 albino guinea pigs received 3 topical induction exposures to 35% (w/w) pine oil in corn oil, with intervals of 5-9 days between these exposures. Two weeks after the third exposure, they were challenged with a 15% w/w suspension at a previously unexposed site, which was scored 24, 48 and 72 hours later. A group of 5 previously unexposed animals were also tested. There were no significant

differences between the two groups with respect to their response. Under the conditions of this study, there was no indication of a dermal sensitization reaction to the test material.

The study adequately indicates a lack of (or very low) dermal sensitization potential for pine oil.

The study is classified as acceptable. This study can be used as supporting data (Guideline 81-6) for the registration and/or reregistration of products consisting of or containing pine oil as an active ingredient.

5. The results of the Ames study demonstrate that **there is no indication of a mutagenic response at the histidine locus in Salmonella typhimurium strains TA98, TA100, TA1535, or TA1537** following exposure to doses ranging from 0.1 to 10000 µg/plate pine oil both in the presence and absence of rat S-9. Some degree of cytotoxicity, as evidenced by reduced numbers of revertants, was observed in all strains at doses ≥100 µg/plate, although there was complete (or nearly complete) cytotoxicity only with strain TA1537 at 10000 µg/plate ±S9.

   This study satisfies the Guideline requirement [84-2(a)] for a gene mutation assay for pine oil, and can be used as supporting data for purposes of registration and/or reregistration. Although dosing solutions were not analyzed, there were sufficient indications of cytotoxicity (reduced numbers of revertants) at the highest doses tested.

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GUIDELINE 81-4

Reviewed by: Byron T. Backus, Ph.D. Byron T. Backus 3/17/95
Section II, Toxicology Branch II (7509C)
Secondary Reviewer: K. Clark Swentzel K. Clark Swentzel 3/17/95
Section II, Tox Branch II (7509C)

DATA EVALUATION REPORT I

STUDY TYPE: Primary Eye Irritation

CHEMICAL: Pine Oil

Tox. Chem. No.: 665 DP Barcode: D210191

PC Code: 067002 Submission: S478447

MRID NUMBER: 433752-09

SYNONYMS/CAS No.: 8002-09-03

SPONSOR: CSMA Pine Oil Joint Venture

TESTING FACILITY: Cosmopolitan Safety Evaluation, Inc.
P.O. Box 71
Lafayette, NJ 07848

TITLE OF REPORT: Pine Oil: Primary Eye Irritation in Rabbits

AUTHOR: Robbins, G. R.

LAB STUDY NUMBER: D3371

STUDY COMPLETION DATE: May 6, 1994

EXECUTIVE SUMMARY: 6/6 rabbit eyes showed corneal opacity in the period between 24 hrs and 4 days after exposure to the test material, with corneal opacity still present in 2/6 eyes at 7 days, and all eyes normal by day 17.

TOXICITY CATEGORY: II

STUDY CLASSIFICATION: Acceptable. The study adequately defines a toxicity category II classification (signal word: WARNING) for Pine Oil in terms of its primary eye irritation potential. This study can be used as supporting data (Guideline 81-4) for the registration and/or reregistration of products consisting of or containing pine oil as an active ingredient.

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PRIMARY EYE IRRITATION IN RABBITS

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A. MATERIALS

1. Test Material: CSMA Pine Oil Blend 012494
   Description: clear liquid
   Lot number: 5558-012494
   Purity:
   Receipt date: February 4, 1994
   Stability: "The stability of the test substance was not tested at C.S.E. and the sponsor will provide such information as appropriate. While at C.S.E. the test substance was stored in the original container at room temperature."

   Contaminants: not reported.
   Other information: "For this study the test substance container was inverted several times and was dosed in the form received without being diluted."

2. Test animals: From information on p. 9 these were six healthy young albino rabbits bred in the laboratory colony and within a weight range of 2.2 to 2.5 kg at the preliminary eye examination or at the time of dosing. From p. 9: "Twenty-four hours prior to dosing, both eyes of each rabbit were examined using a hand held ophthalmoscope and then by an ultraviolet light to detect fluorescein retention. For this procedure one drop of Fluorescein Sodium Ophthalmic Solution USP was dropped onto the cornea and 10-15 seconds later it was washed away with tap water. The eyes were examined for dye retention. Only animals free of apparent ocular defects were selected for this study."

3. Compliance: There is a signed and dated Good Laboratory Practice Compliance Statement on p. 3 of the report, and a signed and dated Quality Assurance Final Report Statement on p. 4.

B. TEST PERFORMANCE AND RESULTS:

1. Dosing: "One eye of each animal was dosed with 0.1 ml of the test substance, while the other eye served as an untreated control."

   "At the time of dosing, the animal was held quiet and the lower lid was everted from the eyeball to form a cup into which the test substance was placed at the specified dose. The eyelids were then gently held together for one second. The eyes were not washed after instillation of the test substance unless, one hour later, the accumulation of debris/discharge interfered with eye examination and this was noted in the observations."

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PRIMARY EYE IRRITATION IN RABBITS

I-3

2. Scoring: "All treated and control eyes were graded and scored at approximately 1, 24, 48 and 72 hours using the criteria established in the "Illustrated Guide for Grading Eye Irritation by Hazardous Substances" which uses a modified Draize scoring method... A hand held ophthalmoscope or slit lamp was used to facilitate examination. In addition, at approximately 24 and 72 hours, both eyes were examined using the fluorescein method as previously described. Since positive ocular scores persisted at 72 hours, those eyes were re-examined on Day 4 using the same criteria and every three or four days thereafter, until the irritation became 'not positive' or the healing was considered likely to be very prolonged. The most serious effects, such as pannus or blistering of the conjunctivae and other effects indicative of corrosive action would have been reported separately."

Results: "Immediately following application of the test substance to the dosed eyes there was no evidence of pain, such as excessive struggling or vocalization; there was temporary blepharospasm."

"At 24 hours there was extensive corneal involvement, iritis and conjunctival irritation. All (6/6) test eyes had opacity (Grade 1) with dye retention over most of the cornea and iritis... All conjunctivae showed redness (Grade 1 or 2) and chemosis (Grade 1 or 2)..."

"By day 4 all eyes still showed opacity (Grade 1 or 2)..."

"By day 7, one eye appeared normal and three eyes were normal except for very slight conjunctival redness (Grade 1 - not positive for irritation). In two eyes there was still corneal opacity (Grade 1 or 2)..."

"On Day 10, four (4/6) eyes were normal except for slight conjunctival redness in one. Two eyes had corneal opacity (Grade 1) with neovascularization..."

"On Day 14 five (5/6) test eyes appeared normal. In one eye there was opacity (Grade 1) with a central area of dye retention and excessive neovascularization; there was no iritis, but there was slight conjunctival redness (Grade 1) and chemosis."

"On Day 17 all test eyes appeared normal and the study was terminated."

C. DISCUSSION AND CONCLUSIONS:

The results of this study (corneal involvement persisting for more than 7 days in some rabbit eyes, with complete healing by day 21) adequately define a toxicity category II classification (signal word: WARNING) for Pine Oil in terms of its primary eye irritation potential. This study can be used as supporting data for the registration and/or reregistration of products consisting of or containing pine oil as an active ingredient.

Primary Eye Irritation. Study #D3371

Table 2a - Ocular Irritation, 1 Hr.

SCORE CHART

Animal # / Test Eye	2896 R	2897 R	2898 R	2899 R	2900 R	2901 R
Cornea						
Opacity	0	1	0	0	0	0
Iris						
Iritis	1	1	1	1	1	1*
Conjunctivae						
Redness	1	1	1	1	1	1
Chemosis	3	2	2	3	2	3
Opaque Area	None	[illegible]	None	None	None	None
Dye Retention	N/A	N/A	N/A	N/A	N/A	N/A
Response:						
Test Eye	Pos	Pos	Pos	Pos	Pos	Pos
Control	Neg	Neg	Neg	Neg	Neg	Neg

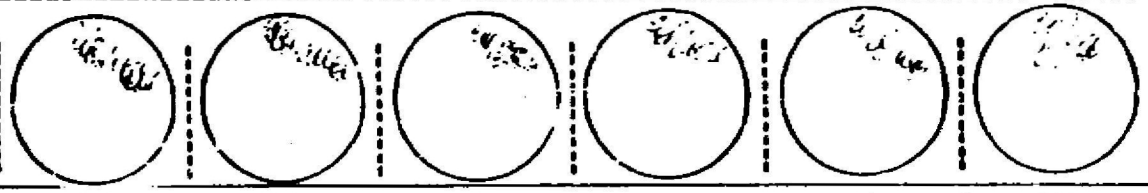
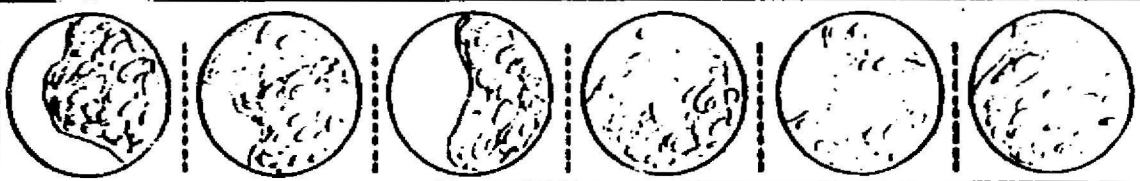
Note: P = Pannus V = Neovascularization (Minimal granulation)
Cornea scored for most severely affected area. 'Neg' indicates the eye was not positive for corneal involvement or irritation. (Blank or absent diagrams indicate no opacity or dye retention at this time.) N/A = Not applicable this period

* Vessels slightly injected

Primary Eye Irritation. Study #D3371

Table 2b - Ocular Irritation, 24 Hr.

SCORE CHART

Animal # / Test Eye	2896 R	2897 R	2898 R	2899 R	2900 R	2901 R
Cornea						
Opacity	1	1	1	1	1	1
Iris						
Iritis	1	1	1	1*	1*	1*
Conjunctivae						
Redness	2D	2	2	1	2	1
Chemosis	2	1	1	1	1	2
Opaque Area						
Dye Retention						
Response: Test Eye	Pos	Pos	Pos	Pos	Pos	Pos
Control	Neg	Neg	Neg	Neg	Neg	Neg

Note: P = Pannus V = Neovascularization (Minimal granulation)
Cornea scored for most severely affected area. 'Neg' indicates the eye was not positive for corneal involvement or irritation.
(Blank or absent diagrams indicate no opacity or dye retention at this time.)

* = Vessels slightly injected
D = Discharge

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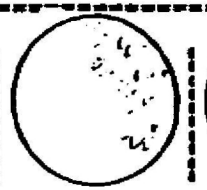
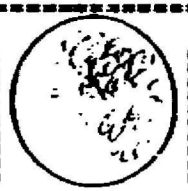
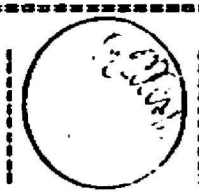
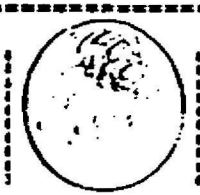
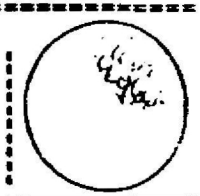
Table 2c - Ocular Irritation, 48 Hr.

SCORE CHART

Animal # / Test Eye	2896 R	2897 R	2898 R	2899 R	2900 R	2901 R
Cornea						
Opacity	1	2	1	2	1	1
Iris						
Iritis	1	1	1	1*	0	0
Conjunctivae						
Redness	2[D]	2	1[D]	1	2	1
Chemosis	2	1	1	1	1	1
Opaque Area						
Dye Retention	N/A	N/A	N/A	N/A	N/A	N/A
Response:						
Test Eye	Pos	Pos	Pos	Pos	Pos	Pos
Control	Neg	Neg	Neg	Neg	Neg	Neg

Note: P = Pannus V = Neovascularization (Minimal granulation)
Cornea scored for most severely affected area. 'Neg' indicates the eye was not positive for corneal involvement or irritation. (Blank or absent diagrams indicate no opacity or dye retention at this time.) N/A = Not applicable this period.

* = Vessels slightly injected
D = Discharge

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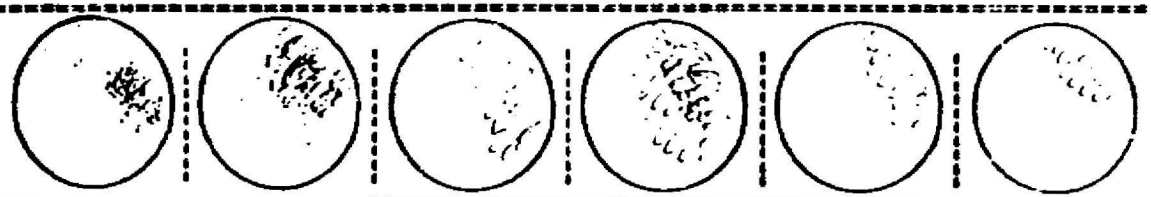
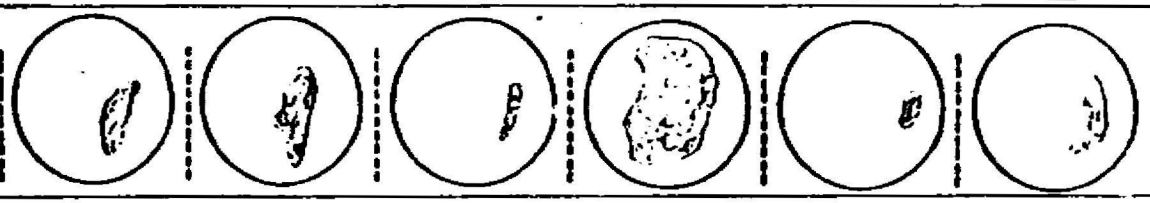
Table 2d - Ocular Irritation, 72 Hr.

SCORE CHART

Animal # / Test Eye	2896 R	2897 R	2898 R	2899 R	2900 R	2901 R
Cornea						
Opacity	2	2	1	2	1	1
Iris						
Iritis	1	1	1	1	0	0
Conjunctivae						
Redness	2	1	1	1	1	1
Chemosis	2	1	1	1	1	1
Opaque Area						
Dye Retention						
Response:						
Test Eye	Pos	Pos	Pos	Pos	Pos	Pos
Control	Neg	Neg	Neg	Neg	Neg	Neg

Note: P = Pannus V = Neovascularization (Minimal granulation)
Cornea scored for most severely affected area. 'Neg' indicates the eye was not positive for corneal involvement or irritation.
(Blank or absent diagrams indicate no opacity or dye retention at this time.)

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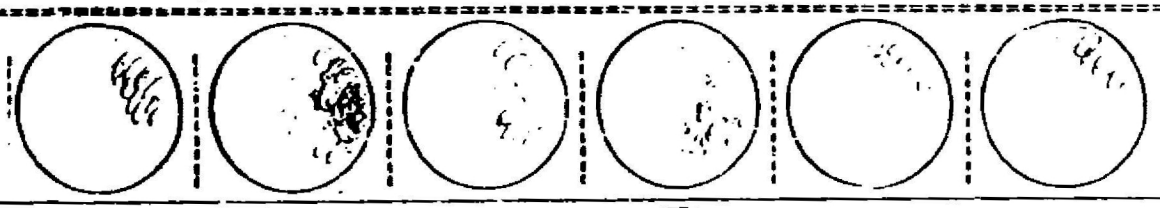
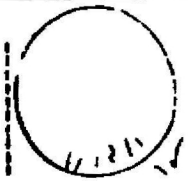
Table 2e - Ocular Irritation, Day 4

SCORE CHART

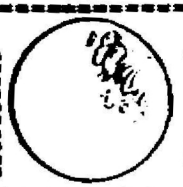
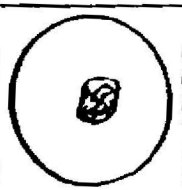
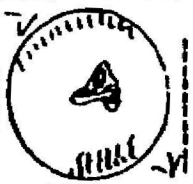
Animal # / Test Eye	2896 R	2897 R	2898 R	2899 R	2900 R	2901 R
Cornea						
Opacity	1	2	1	2	1	1
Iris						
Iritis	1	1	1	1	0	0
Conjunctivae						
Redness	2	1	1	1	1	1
Chemosis	1	1	1	1	1	1
Opaque Area						
Dye Retention	N/A	N/A	N/A		N/A	N/A
Response: Test Eye	Pos	Pos	Pos	Pos	Pos	Pos
Control	Neg	Neg	Neg	Neg	Neg	Neg

Note: P = Pannus V = Neovascularization (Minimal granulation)
Cornea scored for most severely affected area. 'Neg' indicates the eye was not positive for corneal involvement or irritation.
(Blank or absent diagrams indicate no opacity or dye retention at this time.)

Primary Eye Irritation. Study #D3371

Table 2f - Ocular Irritation, Day 7

SCORE CHART

Animal # / Test Eye	2896 R	2897 R	2898 R	2899 R	2900 R	2901 R
Cornea						
Opacity	0	1	0	2	0	0
Iris						
Iritis	0	1	0	1	0	0
Conjunctivae						
Redness	1	1	1	1	1	0
Chemosis	0	1	0	1	0	0
Opaque Area	None		None		None	None
Dye Retention	None		None		None	None
Response: Test Eye	Neg	Pos	Neg	Pos	Neg	Neg
Control	Neg	Neg	Neg	Neg	Neg	Neg

Note: P = Pannus V = Neovascularization (Minimal granulation)
Cornea scored for most severely affected area. 'Neg' indicates the eye was not positive for corneal involvement or irritation.
(Blank or absent diagrams indicate no opacity or dye retention at this time.)

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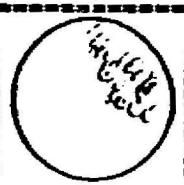
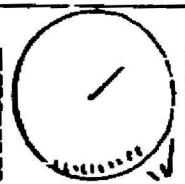
Table 2g - Ocular Irritation, Day 10

SCORE CHART

Animal # / Test Eye	2896 R	2897 R	2898 R	2899 R	2900 R	2901 R
Cornea						
Opacity	0	1	0	1	0	0
Iris						
Iritis	0	1	0	0	0	0
Conjunctivae						
Redness	1	1	0	1	0	0
Chemosis	0	1	0	1	0	0
Opaque Area	None		None		None	None
Dye Retention	None		None		None	None
Response: Test Eye	Neg	Pos	Neg	Pos	Neg	Neg
Control	Neg	Neg	Neg	Neg	Neg	Neg

Note: P = Pannus V = Neovascularization (Minimal granulation)
Cornea scored for most severely affected area. 'Neg' indicates the eye was not positive for corneal involvement or irritation.
(Blank or absent diagrams indicate no opacity or dye retention at this time.)

✓ = indicates examination but no opacity/dye retention at this time

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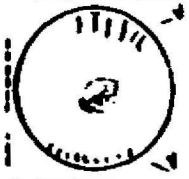
Primary Eye Irritation. Study #D3371

Table 2h - Ocular Irritation, Day 14

SCORE CHART

Animal # / Test Eye	2896 R	2897 R	2898 R	2899 R	2900 R	2901 R
Cornea						
Opacity	0	1	0	0	0	0
Iris						
Iritis	0	0	0	0	0	0
Conjunctivae						
Redness	0	1	0	0	0	0
Chemosis	0	1	0	0	0	0
Opaque Area	None		None	None	None	None
Dye Retention	None		None	None	None	None
Response: Test Eye	Neg	Pos	Neg	Neg	Neg	Neg
Control	Neg	Neg	Neg	Neg	Neg	Neg

Note: P = Pannus V = Neovascularization (Minimal granulation)
Cornea scored for most severely affected area. 'Neg' indicates the eye was not positive for corneal involvement or irritation.
(Blank or absent diagrams indicate no opacity or dye retention at this time.)

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Primary Eye Irritation. Study #D3371

Table 2i - Ocular Irritation, Day 17

SCORE CHART

Animal # / Test Eye	2896 R	2897 R	2898 R	2899 R	2900 R	2901 R
Cornea						
Opacity	0	0	0	0	0	0
Iris						
Iritis	0	0	0	0	0	0
Conjunctivae						
Redness	0	0	0	0	0	0
Chemosis	0	0	0	0	0	0
Opaque Area	None	None	None	None	None	None
Dye Retention	None	None	None	None	None	None
Response: Test Eye	Neg	Neg	Neg	Neg	Neg	Neg
Control	Neg	Neg	Neg	Neg	Neg	Neg

Note: P = Pannus V = Neovascularization (Minimal granulation)
Cornea scored for most severely affected area. 'Neg' indicates the eye was not positive for corneal involvement or irritation.
(Blank or absent diagrams indicate no opacity or dye retention at this time.)

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GUIDELINE 81-5

Reviewed by: Byron T. Backus, Ph.D. Byron T. Backus 3/17/95
Section II, Toxicology Branch II (7509C)
Secondary Reviewer, K. Clark Swentzel K. Clark Swentzel 3/17/95
Section II, Tox Branch II (7509C)

DATA EVALUATION REPORT II

STUDY TYPE: Primary Dermal Irritation

CHEMICAL: Pine Oil

Tox. Chem. No.: 665 DP Barcode: D210191

PC Code: 067002 Submission: S473447

MRID NUMBER: 433752-10

SYNONYMS/CAS No.: 8002-09-03

SPONSOR: CSMA Pine Oil Joint Venture

TESTING FACILITY: Cosmopolitan Safety Evaluation, Inc.
P.O. Box 71
Lafayette, NJ 07848

TITLE OF REPORT: Pine Oil: Primary Dermal Irritation in Rabbits

AUTHOR: Robbins, G. R.

LAB STUDY NUMBER: E3371

STUDY COMPLETION DATE: May 6, 1994

EXECUTIVE SUMMARY: 0.5 ml of the test material was applied to single sites on each of 6 rabbits, with 4 hour occluded exposure. At 72 hours the mean combined score for erythema and edema was 3.33; the PDIS calculated by combining 24 and 72 hour scores was 3.67. All dermal irritation was gone by day 8; indicating no permanent dermal damage.

TOXICITY CATEGORY: III

STUDY CLASSIFICATION: Acceptable. The study adequately defines a toxicity category III classification for Pine Oil in terms of its primary dermal irritation potential. This study can be used as supporting data (Guideline 81-5) for the registration and/or reregistration of products consisting of or containing pine oil as an active ingredient.

011445

PRIMARY DERMAL IRRITATION IN RABBITS

II-2

A. MATERIALS

1. Test Material: CSMA Pine Oil Blend 012494 (80% total terpene alcohols)
   Description: clear liquid
   Lot number: 5558-012494
   Purity: 100% a.i.
   Receipt date: February 4, 1994
   Stability: "The stability of the test substance was not tested at C.S.E. and the sponsor will provide such information as appropriate. While at C.S.E. the test substance was stored in the original container at room temperature."

   Contaminants: not reported. The test material is stated to be (Appendix III) 100% active ingredients.
   Other information: "For this study the test substance container was inverted several times and was dosed in the form received without being diluted." Expiration date is given (Appendix III) as 12/31/96.

2. Test animals: From information on p. 7 these were six healthy young New Zealand White (albino) rabbits bred in the laboratory colony and within a weight range of 2.1 to 2.9 kg at the time of testing. From information on p. 9 the animals had been transferred to the study room with acclimatization for at least 5 days (presumably prior to testing).

3. Compliance: There is a signed and dated Good Laboratory Practice Compliance Statement on p. 3 of the report, and a signed and dated Quality Assurance Final Report Statement on p. 4.

B. TEST PERFORMANCE AND RESULTS:

1. Dosing: From p. 9: "A dose of 0.5 ml of the test substance was applied per application site. The exposure was maintained for 4 hours."

   "On the day before dosing, the dorsal surface around the test site of each rabbit was clipped closely. The skin was gently wiped with a clean moistened paper towel. Any animal having a severe reaction two hours after clipping was not used. One site on each animal was selected for testing. Test substance was introduced under a gauze pad approximately 6 cm^2 secured by hypo-allergenic adhesive tape. The trunk of the rabbit was then wrapped with non-irritating perforated plastic sheeting fastened

at each end with masking tape in order to prevent displacement of the test substance. (Note: Because this method of wrapping is not easily displaced, rabbits were not restrained for the subsequent 4 hour period, diminishing stress on the rabbit and cutting the incidence of spinal injury.) At the end of the 4 hour exposure period, the plastic sheeting and gauze were removed and the skin was wiped with a clean moistened paper towel to remove any test substance still remaining."

2. Scoring: "Thirty to sixty minutes after cleaning the skin at approximately 4 hours, the reactions at each site were scored for erythema and edema using a modification of the method of Draize... Reading were also made at the end of 24, 48 and 72 hours (approximately) and on Day 4 after patch removal. The test substance would be considered as a primary irritant if the primary irritation index was greater than 2.5 (maximum possible score 4)...or if there was scarring, ulceration or full thickness necrosis constituting irreversible tissue destruction."

   Results: Refer to appended page 1 for individual scoring results. The PDIS is 3.67 (average individual score for erythema + edema for 24 and 72 hour readings).

C. DISCUSSION AND CONCLUSIONS:

We can concur with the statement from the laboratory (see p. 12) that: "The EPA toxicity indicator is best described as Category III, moderate irritation at 72 hours." A PDIS of 3.67 (average of erythema + edema for the 24 and 72 hour readings) places the test material in toxicity category III in terms of its dermal irritation potential. This study can be used as supporting data for the registration and/or reregistration of products consisting of or containing pine oil as an active ingredient.

DER II - Appended p

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Primary Dermal Irritation. Study #E3371

Table 4 - Dermal (P.I. Scores)[6]

Time	Animal Number 2890 Er	2890 Ed	2891 Er	2891 Ed	2892 Er	2892 Ed	2893 Er	2893 Ed	2894 Er	2894 Ed	2895 Er	2895 Ed
0.75m	2	2	2	1	1	2	1	1	1	1	2	2
24 hr	2	3	2	2	2	2	2	1	2	2	2	2
48 hr	2	2	2	2	2	2	2	1	2	2	2	1
72 hr	2	2	2Sd	1	2	2	2	1	2Sd	1	2Sd	1
SUM	17		14		15		11		13		14	
+8= SCORE	2.125		1.75		1.875		1.375		1.625		1.75	

MEAN (P.I.): 1.75 ± 0.3

ADDITIONAL DAYS

Time	Animal Number 2890 Er	2890 Ed	2891 Er	2891 Ed	2892 Er	2892 Ed	2893 Er	2893 Ed	2894 Er	2894 Ed	2895 Er	2895 Ed
Day 4	2Sd	1	2Sd	0	2Sd	1	2Sd	0	2Sd	0	2Sd	0
Day 5	2Sd	0	1Sd	0	2Sd	0	1Sd	0	1Sd	0	1Sd	0
Day 6	1Sd	0	0Sd	0	1Sd	0	1Sd	0	0Sd	0	0Sd	0
Day 7	1Sd	0	0Sd	0	1Sd	0	0Sd	0	0Sd	0	0Sd	0
Day 8	0Sd	0	0Sd	0	0Sd	0	0Sd	0	0Sd	0	0Sd	0

Abbreviations (if used) are as follows:

Er = Erythema
Ed = Edema
Es = Eschar
Ne = Necrosis
Pa = Pallor
Bl = Blanching (Erythema around a pale or blanched central area will be shown as score followed by (Pa) or (Bl)
Sd = (Superficial) desquamation
Fi = Fissuring

[6] Draft IRLG Guidelines. March, 1981.

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GUIDELINE 81-6

Reviewed by: Byron T. Backus, Ph.D. Byron T. Backus 3/17/95
Section II, Toxicology Branch II (7509C)
Secondary Reviewer, K. Clark Swentzel K. Clark Swentzel 3/17/95
Section II, Tox Branch II (7509C)

DATA EVALUATION REPORT III

STUDY TYPE: Dermal Sensitization Study (Bühler) in Guinea Pigs

CHEMICAL: Pine Oil

Tox. Chem. No.: 665 DP Barcode: D210191

PC Code: 067002 Submission: S478447

MRID NUMBER: 433752-11

SYNONYMS/CAS No.: 8002-09-03

SPONSOR: CSMA Pine Oil Joint Venture

TESTING FACILITY: Cosmopolitan Safety Evaluation, Inc.
P.O. Box 71
Lafayette, NJ 07848

TITLE OF REPORT: Pine Oil: Dermal Sensitization (Bühler) in Guinea Pigs.

AUTHOR: Robbins, G. R.

LAB STUDY NUMBER: F3371

STUDY COMPLETION DATE: May 6, 1994

EXECUTIVE SUMMARY: 10 albino guinea pigs received 3 topical induction exposures to 35% (w/w) pine oil in corn oil, with intervals of 5-9 days between these exposures. Two weeks after the third exposure, they were challenged with a 15% w/w suspension at a previously unexposed site, which was scored 24, 48 and 72 hours later. A group of 5 previously unexposed animals were also tested. There were no significant differences between the two groups with respect to their response. Under the conditions of this study, there was no indication of a dermal sensitization reaction to the test material.

STUDY CLASSIFICATION: Acceptable. The study adequately indicates a lack of (or low) dermal sensitization potential for pine oil. This study can be used as supporting data (Guideline 81-6) for the registration and/or reregistration of products consisting of or containing pine oil as an active ingredient.

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DERMAL SENSITIZATION IN GUINEA PIGS

III-2

A. MATERIALS

1. Test Material: CSMA Pine Oil Blend 012494 (80% total terpene alcohols)
   Description: clear liquid
   Lot number: 5558-012494
   Purity: 100% a.i.
   Receipt date: February 4, 1994
   Stability: "The stability of the test substance was not tested at C.S.E. and the sponsor will provide such information as appropriate. While at C.S.E. the test substance was stored in the original container at room temperature."

   Contaminants: not reported.
   Other information: "For this study the test substance was freshly prepared prior to each application at the concentrations required for dosing."

2. Test animals: From information on p. 9 these were young adult guinea pigs (480-497 grams) obtained from Camm Research Lab Animals, Wayne, NJ.

3. Compliance: There is a signed and dated Good Laboratory Practice Compliance Statement on p. 3 of the report, and a signed and dated Quality Assurance Final Report Statement on p. 4.

B. TEST PERFORMANCE AND RESULTS:

1. Dose Selection: The test material was not miscible in water. From p. 9: "Five (5) guinea pigs were exposed to four (4) concentrations (10, 25, 50 and 100% w/w in corn oil) of the test material in order to determine (approximately) a highest non-irritating concentration and a concentration that caused minimal irritation. In this screen, both sides of the animal were shaved and sites were exposed to the various concentrations of the material; locations of the concentrations were alternated to avoid site-to-site variations. One test site on each animal was treated with corn oil only." Presumably the amount of suspension at each application site was 0.5 ml.

   From information on p. 14 the corn oil alone caused a slight, patchy erythema (barely perceptible or questionable) in 4/5 animals. 4/5 animals receiving 50% had grade 1 erythema, while 4/5 receiving 25% had a score of ±. Refer to appended page 1 for the scores and scoring system in the dose selection screen.

2. Induction: "Twenty-four hours before first application of the test substance, and subsequently as necessary, hair was closely clipped on the right side of ten animals. A dose of 0.5 ml of the freshly prepared test solution in corn oil was applied to a non-adherent sterile (approximately 6 cm^2) pad...covered by hypo-allergenic cloth tape... The pad was placed on the shaved surface of the animal, and then further occluded with a latex wrap...to ensure adequate contact of the test substance with the skin. The patch remained in place for six (6) hours."

   "This induction procedure was repeated at the same site during the next two weeks for a total of three six (6)-hour exposures. The interval between induction exposures may vary between five (5) and nine (9) days. The animals were then left untreated for approximately two weeks prior to primary challenge..."

3. Challenge: "Two weeks after the last induction, the animals and a naive group were challenged at a virgin site (left side) with 0.5 ml...at the non-irritating concentration at each site. The test substance was applied as for induction. Reactions were scored at approximately 24, 48 and 72 hours after challenge."

   Results: Refer to appended pages 2 (principal reader) and 3 (second reader) for individual scoring results at 24 hours. Scores at 48 and 72 hours generally showed reduced irritation in comparison with the 24-hour results.

4. Recent positive control data: The report includes (p. 24-44) the positive results of a dermal sensitization assay in guinea pigs conducted using 1,4-phenylenediamine dihydrochloride. This study was initiated on 24 January 1994 and was completed 6 May 1994, about the same time the sensitization study with pine oil was conducted.

C. DISCUSSION AND CONCLUSIONS:

We can concur with the statement from the laboratory (see p. 7) that: "As a result of this test, CSMA PINE OIL BLEND was not considered a sensitizer in guinea pigs."

The study adequately indicates a low dermal sensitization potential for pine oil. This study can be used as supporting data (Guideline 81-6) for the registration and/or reregistration of products consisting of or containing pine oil as an active ingredient.

DER III - Appended p. 1

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Dermal Sensitization - (Bühler). Study #F3371

Table 1 - Dose Selection Screen

Dilutions:

Site:	A	100 %	- mg in	-
	B	50 %	5.000 mg in	10.000 mg corn oil W/V
	C	25 %	2.500 mg in	10.000 mg corn oil W/V
	D	10 %	1.000 mg in	10.000 mg corn oil W/V
	E	0 %		corn oil

Readings at 24 Hours

Site/Conc	A 100 %	B 50 %	C 25 %	D 10 %	E 0 %
G-Pig #	Grade	Grade	Grade	Grade	Grade
1	2	1	±	±	0
2	3	1	1	1	±
3	2	1	±	±	±
4	1	±	±	±	±
5	2	1	±	±	±
6	-	-	-	-	-

0 = No reaction
± = Slight, patchy erythema (barely perceptible or questionable)
1 = Slight, but confluent or moderate but patchy erythema
2 = Moderate erythema
3 = Severe erythema with or without edema

Guinea-pig Identity: #4343 (1) - #4347 (5/6)

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Dermal Sensitization - (Bühler). Study #P3371

Table 4A - Skin Scores - 24 Hours - Principal Reader

Date Application Reading	Induction Applications First Grade	Second Grade	Third Grade	Challenge Application Virgin Side Grade	Re-Challenge Site Grade
G-pig #					
1	1	1	1	±	-
2	±	1	1	±	-
3	1	1	1	±	-
4	±	1	±	±	-
5	±	1	1	1	-
6	±	1	1	±	-
7	1	1	1	1	-
8	1	1	1	±	-
9	±	1	1	±	-
10	1	1	1	±	-

Naive Control	G-pig #	
	N1	±
	N2	1
	N3	±
	N4	±
	N5	±

Scoring:

0 = No reaction
± = Slight, patchy erythema (barely perceptible or questionable)
1 = Slight, but confluent or moderate but patchy erythema
2 = Moderate erythema
3 = Severe erythema with or without edema

Guinea-pig Identity: Test Group: #4353 (1) .. 4362 (10)
Naive Group: #4363 (N1) .. 4367 (N5)

DER III - Appendix F, 7

Dermal Sensitization - (Bühler). Study #F3371

Table 4B - Skin Scores - 24 Hours - Second Reader

Date Application Reading	Induction Applications First Grade	Second Grade	Third Grade	Challenge Application Virgin Side Grade	Re-Challenge Site Grade
G-pig #					
1	1	1	1	±	-
2	±	1	1	±	-
3	±	1	1	±	-
4	±	1	±	±	-
5	±	1	1	1	-
6	±	1	1	±	-
7	1	1	1	1	-
8	1	1	1	±	-
9	±	1	1	±	-
10	1	1	1	±	-

Naive Control	G-pig #	
	N1	±
	N2	1
	N3	±
	N4	±
	N5	±

Scoring:

0 = No reaction
± = Slight, patchy erythema (barely perceptible or questionable)
1 = Slight, but confluent or moderate but patchy erythema
2 = Moderate erythema
3 = Severe erythema with or without edema

Guinea-pig Identity: Test Group: #4353 (1) .. 4362 (10)
Naive Group: #4363 (N1) .. 4367 (N5)

011445

GUIDELINE 81-3

Reviewed by: Byron T. Backus, Ph.D. Byron T. Backus 3/17/95
Section II, Toxicology Branch II (7509C)
Secondary Reviewer: K. Clark Swentzel M. Clark Swentzel 3/17/95
Section II, Tox Branch II (7509C)

DATA EVALUATION REPORT IV

STUDY TYPE: Acute Inhalation LC50 in Rats

CHEMICAL: Pine Oil

Tox. Chem. No.: 665 DP Barcode: D210191

PC Code: 067002 Submission: S478447

MRID NUMBER: 433752-08

SYNONYMS/CAS No.: 8002-09-03

SPONSOR: CSMA Pine Oil Joint Venture

TESTING FACILITY: Cosmopolitan Safety Evaluation, Inc.
P.O. Box 71
Lafayette, NJ 07848

TITLE OF REPORT: Pine Oil: Acute Inhalation Toxicity Study in Rats

AUTHOR: Robbins, G. R.

LAB STUDY NUMBER: C3371

STUDY COMPLETION DATE: May 6, 1994

EXECUTIVE SUMMARY: There were no mortalities among 10 rats (5 males, 5 females) which received a 4-hour full-body exposure to a measured concentration of 3.79 mg/liter (nominal concentration: 9.2 mg/liter) of Pine Oil. A mean of 40% (by weight) of the particles had a mean aerodynamic diameter <1 micron. Symptoms (depressed activity, yellow perineal and abdominal staining, chromorhinorrhea, ataxia, nasal discharge) were gone by two days after exposure, and respiratory tracts appeared normal at necropsy on day 14. The inhalation LC50 is greater than 3.79 mg/liter.

TOXICITY CATEGORY: IV

STUDY CLASSIFICATION: Acceptable. The study adequately defines a toxicity category IV classification (using the Interim EPA Policy for Particle Size and Limit Concentration Issues in Inhalation Toxicity Studies) for pine oil in terms of its inhalation toxicity

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potential. This study is acceptable as supporting data for purposes of registration and/or reregistration of products consisting of or containing pine oil.

A. MATERIALS

1. Test Material: CSMA Pine Oil Blend 012494 (80% total terpene alcohols)
   Description: clear liquid
   Lot number: 5558-012494
   Date of manufacture: 1-24-94
   Purity: 100% a.i.
   Receipt date: 2-4-94
   Stability: "The stability of the test substance was not tested at C.S.E. and the sponsor will provide such information as appropriate. While at C.S.E. the test substance was stored in the original container at room temperature."

   Contaminants: not reported.
   Other information: "For use in this study the test substance container was inverted several times to thoroughly mix the contents which were poured into the nebulizer container."

2. Test animals: From information on p. 8 these were young adult Sprague-Dawley derived rats bred in the laboratory colony; males were between 310 and 336 grams and the females were between 259 and 277 grams.

3. Compliance: There is a signed and dated Good Laboratory Practice Compliance Statement on p. 3 of the report, and a signed and dated Quality Assurance Final Report Statement on p. 4.

B. TEST PERFORMANCE AND RESULTS:

1. Exposure chamber and delivery system: From information on p. 9 the plexiglass exposure chamber was approximately 50.5 cm long x 29.5 cm high (semicylindrical), with a volume of 47.4 liters. "A wire mesh floor was raised 5.0 cm from the floor of the chamber and on this the rats were individually caged in all wire-mesh cages. The total "volume" of the test animals was not more than 5% of the volume of the test chamber."

   The test substance was delivered from a DeVilbiss Glass Nebulizer; air flow and delivery involved an air pump. "In addition to the four hour exposure period, a period of time (t_{99}) was allowed to compensate for the time required for delivery system stabilization to a nominal chamber concentration greater than 5 mg of test substance per liter of air."

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ACUTE INHALATION IN RATS

IV-3

2. Measurement of concentrations: From p. 12: "The nominal exposure concentration was 9.2 mg/liter, calculated for the stabilized system using the weight of the test substance aerosolized into a known volume of air. [The amount of prepared test substance aerosolized and delivered in the 4-hr exposure period was 22.1 g. (Starting weight of test substance = 786.6 g. Ending weight = 746.5 g.) Total air flow was 2400 liters. Exposure concentration is given by 22.1 g ÷ 2400 liters = 9.2 mg/liter.]" From p. 10: "The actual concentration was determined thrice by gravimetric measurement, using an aerosol analysis monitor with two superimposed filters. The area between the filters was packed with activated charcoal. The atmospheric sample from the breathing zone was drawn through the filters by vacuum filtration at a sampling rate of ±1 liter/minute for not more than ten minutes. Difference in the weight of the monitor before and after filtration was the weight of test substance in the sampled volume of atmosphere that was deposited on the filters and absorbed by the charcoal."

From information on p. 12 the following gravimetric concentrations were measured:

Time	Gain (mg)/10 liters	Actual concentration (mg/l)
1 hr. 1 min.	39.8	3.98
2 hr. 16 min.	39.2	3.92
3 hr. 18 min.	34.8	3.48

"Comparison of the nominal concentration and the actual indicate a dosing efficiency of approximately 41%. The very cloudy atmosphere in the test chamber and the precipitation evident on exposed surfaces indicates that the maximum attainable concentration of aerodynamic particles had been reached or exceeded."

3. Particle size: From p. 13: "Particle sizing was carried out twice during exposure, at 1 hour 32 minutes and at 3 hours 35 minutes. The mass median diameter was determined to be 1.5 microns at approximately 1-1/2 hours and 1.1 microns at approximately 3-1/2 hours. The geometric standard deviation was determined to be ±2.6 and ±3.0 microns, respectively. Approximately 34% and 40% (mean 40%) of the particles had an aerodynamic diameter of <1 micron." Refer to appended page 1 for particle size determinations.

4. Observations on animals: From p. 11: "Animals were observed from outside the chamber for mortality and pharmacotoxic signs hourly during the exposure period. Rats were observed immediately following the t_{99} period after they had been returned to standard

cages and then at approximately 1, 3 and 5 hours following the exposure, and once daily thereafter. In addition, mortality was checked each afternoon (except weekends and holidays) for the remainder of the 14 day observation period."

"Body weights were obtained on Day 0 (before dosing) and on survivors on Days 2, 3, 4, 7 and 14."

"At the end of the observation period, all surviving animals were sacrificed humanely. A postmortem examination of each animal...was carried out with detailed examination of the nasal air passages, trachea, bronchii and lungs..."

Results: "The test substance was visible as it entered the exposure chamber as a fine mist delivered from the portal. The atmosphere in the test substance chamber rapidly became very cloudy and remained very cloudy throughout the exposure period. The walls and floor became wet within one hour and remained wet, presumably due to deposition of the test substance on these surfaces since, except for feces and urine on the floor, the control chamber remained dry."

From p. 13: "Control rats remained normal in appearance and behavior during exposure and during the subsequent 14-day observation period."

"Rats exposed to the test substance remained apparently normal during the first two hours of the exposure period except for the dorsal fur, and later the muzzles, appearing wet. From the third hour all rats had a diminished startle response to tapping on the chamber wall. On return to their cages all rats appeared wet and the common signs were depressed activity, yellow abdominal and perineal staining (urine), and frequent chromorhinorrhea (3♂; 2♀); one female showed ataxia and also had nasal discharge (yellow). For the remainder of the day most rats showed normal activity, but perineal staining (yellow) and frequent chromorhinorrhea persisted; one female continued depressed and had nasal discharge (yellow). By Day 1 all rats had apparently returned to normal on cageside observation except for nasal discharge (yellow) in one male and two female rats."

"From Day 2 all rats appeared normal on cageside examination."

Body weights: Most of the exposed rats lost weight in the two days following exposure, while the controls were gaining weight. However, most of the exposed rats were back to their starting weight by Day 3 or 4 and then gained weight. At termination, exposed rats had essentially the same weight gains as their controls. Refer to appended page 2 for individual and mean body weight changes.

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Necropsy: There were no indications of any effect. All respiratory tracts appeared normal on necropsy.

C. DISCUSSION AND CONCLUSIONS:

We can accept the laboratory's finding that the inhalation LC50 for pine oil is >3.79 mg/liter, as there were no mortalities among 10 rats exposed to this concentration for 4 hours. It is noted that these rats received a full body exposure to the test material, and that their cumulative exposure (which would have included ingestion of the condensed test material while grooming, as well as that from inhalation) would have been greater than that which would have occurred if they had "muzzle only" exposure.

The test material (Pine Oil) has an inhalation LC50 > 2 mg/liter. This defines a Toxicity Category IV hazard potential by this exposure route using the revised inhalation toxicity category classification of the Interim EPA Policy for Particle Size and Limit Concentration Issues in Inhalation Toxicity Studies.

DER IV - Appended p. 1

011445

Acute Inhalation Toxicity. Study #C3371

Table 5 - Particle Size Determinations

A. 1 Hr. 32 Min.

MICRON	Slide Weight (mg) PRE-WT	POST-WT	GAIN	%	CUM.%
>13	1311.4	1313.8	2.4	5.0	100.0
13	1307.9	1313.2	5.3	11.1	95.0
4	1308.1	1322.0	13.9	29.1	83.9
1.7	1304.2	1323.2	19.0	39.8	54.7
0.5	1410.4	1417.5	7.1	14.9	14.9
	TOTAL GAIN		47.7	100.0	

B. 3 Hr. 35 Min.

MICRON	Slide Weight (mg) PRE-WT	POST-WT	GAIN	%	CUM.%
>13	1299.7	1300.2	0.5	1.3	100.0
13	1277.8	1281.1	3.3	8.7	98.7
4	1282.6	1296.3	13.7	36.2	89.9
1.7	1276.6	1287.2	10.6	28.0	53.7
0.5	1415.2	1424.9	9.7	25.7	25.7
	TOTAL GAIN		37.8	100.0	

FROM GRAPHS:

	MASS MEDIAN DIAMETER	GEOMETRIC S.D. (±)	% ≤1 micron
A	1.5 microns	2.6	34
B	1.1 microns	3.0	46

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DER IV - Appendix p. 2

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Acute Inhalation Toxicity. Study #C3371

Table 9 - Body Weights (Grams)

Controls

ANIMAL #	0	2	3	4	7	14	GAIN/LOSS 0-2	GAIN/LOSS 0-14
Males								
7847	335	340	343	350	356	381	5	46
7848	317	321	322	328	340	369	4	52
7849	325	330	332	340	347	366	5	41
7850	330	336	342	349	360	371	6	41
7851	331	335	339	343	354	367	4	36
MEAN	328	332	336	342	351	371	5	43
SD ±	7	7	9	9	8	6	1	6
Females								
7852	265	267	268	274	279	285	2	20
7853	262	266	270	270	275	281	4	19
7854	259	261	264	265	268	276	2	17
7855	270	272	273	277	283	289	2	19
7856	274	275	277	279	280	291	1	17
MEAN	266	268	270	273	277	284	2	18
SD ±	6	5	5	6	6	6	1	1

Nominal Dose >5 mg/liter/4 hours

ANIMAL #	0	2	3	4	7	14	GAIN/LOSS 0-2	GAIN/LOSS 0-14
Males								
7857	335	336	341	347	354	382	1	47
7858	320	307	318	328	332	356	-13	36
7859	310	299	311	318	328	351	-11	41
7860	336	342	344	358	362	386	6	50
7861	316	307	311	322	327	356	-9	40
MEAN	323	318	325	335	341	366	-5	43
SD ±	12	19	16	17	16	16	8	6
Females								
7862	277	275	279	285	288	295	-2	18
7863	264	258	258	258	268	280	-6	16
7864	268	261	263	269	271	282	-7	14
7865	261	253	256	258	267	275	-8	14
7866	275	278	277	277	280	294	3	19
MEAN	269	265	267	269	275	285	-4	16
SD ±	7	11	11	12	9	9	5	2

011445

Guideline Series 84: **MUTAGENICITY**

Reviewed by: Byron T. Backus, Ph.D. Byron T. Backus 3/15/95
Section II, Toxicology Branch II (7509C)
Secondary reviewer: K. Clark Swentzel K. Clark Swentzel 3/17/95
Section II, Tox Branch II (7509C)

DATA EVALUATION REPORT V

STUDY TYPE: Salmonella typhimurium/mammalian microsome mutagenicity (Ames) assay

CHEMICAL: Pine Oil

Tox. Chem. No.: 665 DP Barcode: D210191

PC Code: 067002 Submission: S478447

MRID NUMBER: 433752-12

SYNONYMS/CAS No.: 8002-09-03

SPONSOR: CSMA Pine Oil Joint Venture

TESTING FACILITY: Toxikon Corporation
225 Wildwood Ave.
Woburn, MA 01801

TITLE OF REPORT: Pine Oil: Salmonella Typhimurium Reverse Mutation Assay

AUTHOR: Ferrante, S.

STUDY NUMBER: 94G-0332

STUDY COMPLETION DATE: April 11, 1994

EXECUTIVE SUMMARY:

The results of this study demonstrate that there is **no indication of a mutagenic response at the histidine locus in Salmonella typhimurium strains TA98, TA100, TA1535, or TA1537** following exposure at doses ranging from 0.1 to 10000 µg/plate pine oil both in the presence and absence of rat S-9. Some degree of cytotoxicity, as evidenced by reduced numbers of revertants, was observed in all strains at doses ≥100 µg/plate, although there was complete (or nearly complete) cytotoxicity only with strain TA1537 at 10000 µg/plate ±S9.

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STUDY CLASSIFICATION:

This study satisfies the Guideline requirement [84-2(a)] for a gene mutation assay for pine oil, and can be used as supporting data for purposes of registration and/or reregistration. Although dosing solutions were not analyzed, there were sufficient indications of cytotoxicity (reduced numbers of revertants) at the highest doses tested.

A. MATERIALS

1. Test Material: Pine Oil@100% (CSMA Pine Oil Blend 012494). Batch #: 5558-012494 ratio); Batch #B46-44-1], CAS/Code #: 8002-09-3
Described as a liquid, which was stored "in the original closed container away from heat or sources of ignition in a well ventilated area."
Stability: not reported (p. 10: "The sponsor was responsible for all test substance characterization and stability data as specified in the GLP regulations, Part 160.105).
Contaminants: Not reported
Solvent used: Acetone

2. Control Materials:
Solvent/final concentration: 100 µl/plate
Positive: Non-activation:

Sodium azide	100.0	µg/ml	TA100
Sodium azide	5.0	µg/ml	TA1535
2-Nitrofluorene	10.0	µg/ml	TA98
9-Aminoacridine	800.0	µg/ml	TA1537

Activation:
2-Aminoanthracene

5.0	µg/ml	TA98
10.0	µg/ml	TA100
20.0	µg/ml	TA1535
30.0	µg/ml	TA1537

"The appropriate concentrations for all positive control substances were dosed at 100 µl/plate."

3. Activation: (see p. 14 of the report) S9 derived from:
X Aroclor 1254 X induced X rat X liver
___ phenobarbital ___ non-induced ___ mouse ___ lung
___ none ___ hamster ___ other
___ mixed phenobarbital and 3-methylcholanthrene

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S9 mix composition (from p. 14):

0.4M $MgCl_2$/1.65M KCl	0.20 ml
1M glucose-6-phosphate	0.05 ml
0.1M NADP	0.40 ml
0.2M phosphate buffer Ph 7.4	5.00 ml
"USP water for injection"	3.35 ml
S9 Fraction	1.00 ml

4. Test organisms: S. typhimurium strains
___ TA97 _X_ TA98 _X_ TA100 ___ TA102 ___ TA104
_X_ TA1535 _X_ TA1537 ___ TA1538 ; list any others:

Properly maintained? Yes
Checked for appropriate genetic markers (rfa mutation, R factor)? Yes (p. 13: "The requirement of histidine for growth was demonstrated for each strain. Other phenotypic characteristics were verified by a crystal violet sensitivity and resistance to ampicillin test method. Spontaneous reversion frequency was in the range expected either as reported in literature, or as established by Toxikon's historical mean values).

"Working stock cultures were grown fresh for the assay. Frozen stock of each strain was thawed and inoculated into sterile nutrient broth. Cultures were incubated overnight at 37±2°C. Cultures with an Absorbance >1 at 650 nm, read against a nutrient broth blank, were used in the assay."

5. Test compound concentrations used (µg/plate): From p. 15: "A range finding assay was performed without metabolic activation to determine the levels at which the test substance exhibited toxicity. The assay was conducted only with TA100 and negative control substance plates..."

It is reported (p. 16) that the test substance "was slightly toxic to the cells" at 10,000 and 1,000 µg/plate.

The concentrations tested in the mutagenicity assay were 0.1, 1, 10, 100, 1000 and 10000 µg/plate both with and without S9 activation.

6. Evaluation criteria for a valid assay: "Results for a strain would have been rejected if the positive control article did not yield a mutagenic response."

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7. Evaluation criteria for a positive response: From p. 16: "The mean number of revertants per plate and the standard deviation between triplicate plates were calculated for each concentration and strain. A positive result for any strain would have been a significant increase over the negative control article number of revertants per plate, which was concentration dependent. A significant increase would have been at least a two-fold increase over the number of revertant colonies associated with the corresponding negative control article..."

"The demonstration of a dose-related increase in revertant counts was an important criterion in establishing mutagenicity."

8. There is a signed and dated Good Laboratory Practice Statement on page 3 of the report, and a signed and dated Quality Assurance Statement on p. 6 of the report.

B. TEST PERFORMANCE AND RESULTS:

1. Type of Salmonella assay:
   - _x_ standard plate test
   - ___ pre-incubation (___ minutes)
   - ___ "Prival" modification (i.e. azo reduction method)
   - ___ spot test

2. Preliminary cytotoxicity assay: A range-finding assay was performed using strain TA100, but only in the absence of S9 (for results refer to p. 18). There were decreases in numbers of revertants at both 1000 and 10,000 µg/plate, but not at doses ranging from 0.1 to 100 µg/plate.

3. Mutagenicity assays: Refer to appended pages 1 and 2 for the results of the assays. There was no indication of a mutagenic response in any of the strains used, and there were indications of cytotoxicity (reduced numbers of revertants) in all strains at the two highest dose levels (1000 and 10000 µg/plate) ±S9; and in some of the strains at 100 µg/plate. The reporting does not state whether or not there were other indications of cytotoxicity (such as reduced background lawn) at these dose levels. The positive controls elicited appropriate responses.

C. DISCUSSION AND CONCLUSIONS:

While there are some minor flaws evident in the reporting and/or conduct of this study (no analyses of dosing solutions, no reporting as to whether or not there were indications of cytotoxicity other than a reduction in number of revertants) it is concluded that there is sufficient information to demonstrate

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that exposure to the test material, 100% pine oil, under the conditions of this assay, does not cause a mutagenic response in Salmonella typhimurium strains TA98, TA100, TA1535, or TA1537 either in the presence or absence of S9 activation.

This study satisfies the Guideline requirement [84-2(a)] for a gene mutation assay for Pine Oil, and can be used as supporting data for purposes of registration and/or reregistration. Although dosing solutions were not analyzed, the test material was assayed to (and over a sufficiently adequate range of) cytotoxic concentrations.

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TABLE II

Reverse Mutation Assay
Without Microsomal Activation

Test Article:
CSMA Pine Oil Blend 012494

Technical Initiation: 03/18/94

Vendor Lot #:
5558-012494

Technical Completion: 03/21/94

Revertants/Plate*

	CONTROLS		TEST ARTICLE					
STRAIN	Positive Control**	Negative Control***	Dose Levels (ug/plate) 10000	1000	100	10	1	0.1
	189	27	5	13	18	22	24	25
TA98	192	25	8	13	22	19	20	24
	195	26	7	10	17	19	25	22
MEAN	192.0	26.0	6.7	12.0	19.0	20.0	23.0	23.7
SD	3.0	1.0	1.5	1.7	2.6	1.7	2.6	1.5
	357	106	40	43	71	109	123	124
TA100	368	110	40	41	72	111	129	132
	374	118	39	45	75	114	124	126
MEAN	366.3	111.3	39.7	43.0	72.7	11.33	125.3	127.3
SD	8.6	6.1	0.6	2.0	2.1	2.5	3.2	4.2
	114	18	5	10	12	16	19	16
TA1535	118	19	4	8	13	18	17	18
	122	18	5	12	15	19	20	19
MEAN	118.0	18.3	4.7	10.0	13.3	17.7	18.7	17.7
SD	4.0	0.6	0.6	2.0	1.5	1.5	1.5	1.5
	201	8	0	2	3	5	9	10
TA1537	212	8	0	0	2	8	6	8
	208	7	0	7	1	6	8	6
MEAN	207.0	7.7	0.0	3.0	2.0	6.3	7.7	8.0
SD	5.6	0.6	0.0	3.6	1.0	1.5	1.5	2.0

* All plates were dosed at 100 ul/plate
** The positive control used was sodium azide for strains TA-100 and TA-1535, 2-nitrofluorene for strain TA-98, and 9-aminoacridine for strain TA-1537
*** The negative control used in the assay was Acetone

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TABLE III

Reverse Mutation Assay
With Microsomal Activation

Test Article:
CSMA Pine Oil Blend 012494

Technical Initiation: 03/18/94

Vendor Lot #:
5558-012494

Technical Completion: 03/21/94

Revertants/Plate*

	CONTROLS		TEST ARTICLE					
STRAIN	Positive Control**	Negative Control***	Dose Levels (ug/plate) 10000	1000	100	10	1	0.1
	247	34	10	14	24	33	32	31
TA98	252	35	10	17	27	34	35	38
	251	37	9	19	28	32	34	36
MEAN	250.0	35.3	9.7	16.7	26.3	33.0	33.7	35.0
SD	2.6	1.5	0.6	2.5	2.1	1.0	1.5	3.6
	450	128	50	58	98	117	127	136
TA100	439	132	48	60	105	122	128	129
	444	131	43	57	101	123	131	138
MEAN	444.3	130.3	47.0	58.3	101.3	120.7	128.7	134.3
SD	5.5	2.1	3.6	1.5	3.5	3.2	2.1	4.7
	218	22	8	10	15	26	26	27
TA1535	227	29	8	12	15	23	20	26
	221	24	6	13	19	21	25	24
MEAN	222.0	25.0	7.3	11.7	16.3	23.3	23.7	25.7
SD	4.6	3.6	1.2	1.5	2.3	2.5	3.2	1.5
	287	10	0	1	6	11	11	14
TA1537	269	12	0	3	8	13	10	12
	270	14	1	1	10	12	14	13
MEAN	275.3	12.0	0.3	1.7	8.0	12.0	11.7	13.0
SD	10.1	2.0	0.6	1.2	2.0	1.0	2.1	1.0

* All plates were dosed at 100 ul/plate
** The positive control used was 2-aminoanthracene for all strains
*** The negative control used in the assay was Acetone

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